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Influence of age on nitric oxide modulatory action on Na^+, K^+-ATPase activity through cyclic GMP pathway in proximal rat trachea

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Abstract

Age-related changes in the modulatory action of nitric oxide (NO) on cyclic GMP levels and Na^+,K^+-ATPase activity in the proximal rat trachea were investigated using sodium nitroprusside, 8-bromo-cyclic GMP and okadaic acid. At 24 months, both control activities of Na^+,K^+-ATPase and Mg^{2+}-ATPase were decreased when compared to the segments from 4- and 12-month-old animals. However, cyclic GMP levels were similar among the three ages. Sodium nitroprusside (100 μM) induced stimulation of Na^+,K^+-ATPase activity in segments from both 4- and 12-month-old animals, but not 24-month-old animals. The effect was specific for Na^+,K^+-ATPase since Mg^{2+}-ATPase activity was unaffected. Sodium nitroprusside induced an increase in nitrates/nitrites and cyclic GMP levels in proximal segments at 4, 12 and 24 months. The 8-bromo-cyclic GMP (100 μM) induced a similar specific stimulation of Na^+,K^+-ATPase activity in segments from 4- and 12- but not 24-month-old animals. Okadaic acid (1 μM), a phosphatase-1 inhibitor, increased proximal Na^+,K^+-ATPase but not Mg^{2+}-ATPase activity in tissues from 4-, 12- and 24-month-old animals. Our results suggest that aging affects cyclic GMP pathway in proximal rat trachea by an action at the level of the cyclic GMP-dependent protein kinase.

Keywords: Nitric oxide (NO); cGMP-dependent protein kinase; Na^+,K^+-ATPase; Aging; Trachea, rat

1. Introduction

According to the free radical theory, one of the most prominent current theories of aging, altered mitochondrial metabolism can cause cell death and abnormal function (Beal et al., 1993). Regarding this theory, several reports suggest that the increase in nitric oxide (NO) in the pineal gland and anterior pituitary may be an important factor in aging (McCann, 1997; McCann et al. 1998). In addition, aging modifies the *N*-methyl-D-aspartate (NMDA)-NO-cyclic GMP pathway in rat central nervous system (Peterson and Cotman, 1989; Tamaru et al., 1991; Wenk et al. 1991; Cimino et al. 1993; Lipton, 1994; Vallebuona and Raiteri, 1995).

Smooth muscle relaxation induced by NO-generating compounds has been considered to be mediated by the increase in cyclic GMP, formed through NO catalytic action on soluble guanylyl cyclase, which in turn, activates cyclic GMP-dependent protein kinase (Katsuki and Murad, 1977; Murad et al., 1978; Buga et al., 1989; Gruetter et al., 1989; Nijkamp and Folkerts, 1994, 1995; Torphy, 1994; Folkerts et al., 1995).

Relaxation of airways smooth muscle of several species elicited through non-cholinergic-non-adrenergic innervation is thought to be mediated by NO (Katsuki and Murad, 1977; Murad et al., 1978; Buga et al., 1989; Gruetter et al., 1989; Li and Rand, 1991; Belvisi et al., 1992; Ellis and Undem, 1992; Nijkamp and Folkerts, 1994). In addition, NO is also involved in airway inflammation and, therefore, it is assumed that NO plays a double role in the regulation of physiological and pathophysiological processes of airways (Barnes and Belvisi, 1993; Belvisi et al., 1995).

Na^+,K^+-ATPase is a member of a family of transmembrane spanning enzymes consisting of two different poly-

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peptides, α (catalytic) and β subunits. In the rat, three isoforms of α subunit and three of β subunit have been identified (Sweadner, 1989; Arystarkhova and Sweadner, 1997; Besirli et al., 1997). Recently, we demonstrated that NO, through generation of cyclic GMP and stimulation of cyclic GMP-dependent protein kinase, plays an important role in the modulation of Na^+,K^+-ATPase activity in the proximal segment of rat trachea (De Oliveira Elias et al., 1999). Studies of the non-adrenergic non-cholinergic-NO/cyclic GMP pathway in muscle strips from the gastric fundus have demonstrated that the relaxant responses to sodium nitroprusside, 8-bromo-cyclic GMP and zaprinast were reduced with age, although sodium nitroprusside induced a similar elevation of the cyclic GMP content in tissue from 3-, 12- and 24-month-old animals (Smits and Lefebvre, 1995).

Although chronic inflammatory pulmonary disease is one of the most common respiratory diseases in advanced life, the airway system has received little attention in experimental studies on aging (Escolar et al., 1995; Rossi et al., 1996; Renwick and Connolly, 1999). In order to investigate the age-dependent alterations in the NO/cyclic GMP pathway in the proximal segment of rat trachea, we studied changes in the effects of sodium nitroprusside, 8-bromo-cyclic GMP and okadaic acid on Na^+,K^+-ATPase activity in proximal trachea from 4-, 12- and 24-month-old animals. In addition, the effect of sodium nitroprusside on cyclic GMP and nitrite/nitrate levels of the proximal segments of rat trachea in the three age groups was also evaluated.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (4-, 12- and 24-month-old) were killed with an overdose of chloral hydrate (> 400 mg/kg, i.p.), exsanguinated and the thorax was cut open. The trachea was removed and dissected free of adherent connective tissue. The proximal (corresponding to three to five first cartilaginous rings close to the larynx) segments from 10 animals from each group were pooled, and slices ($0.3 \times 0.3 \times 1$ mm) were prepared on a Brinkmann tissue chopper, washed extensively to remove small particles, cooled to 4°C and resuspended (15 mg/ml) in buffer containing (in mmol/l): NaCl, 137; KCl, 5; $MgSO_4$, 0.8; $CaCl_2$, 1.0; HEPES, 10; and NaOH to adjust the pH to 7.4 at 34°C.

2.2. Measurement of Na^+,K^+-ATPase activity

The drugs (sodium nitroprusside, 8-bromo-cyclic GMP, okadaic acid) were added (10 μl) to tubes containing 990-μl aliquots of the tissue preparation (five replicates), incubated for 15 min at 34°C, and then rapidly frozen on dry ice to stop the reaction. The samples were thawed, centrifuged at $3700 \times g$ for 15 min, and the supernatants were removed, heated at 90°C for 5 min to prevent degradation of cyclic GMP, and kept at −80°C for cyclic GMP assay. A fresh reaction buffer (1.0 ml of ATPase reaction buffer containing, in mmol/l: NaCl, 85; KCl, 20; $MgCl_2$, 4; EGTA, 0.2; histidine, 30; and NaOH 10 N, approximately 20 μl to adjust to pH 7.2 at 34°C) was added to each tube. The tissue was homogenised, centrifuged at $3700 \times g$ for 15 min, and the supernatant was removed and similar new fresh buffer was added, followed by the addition of a V_{max} concentration (10 mM) of ATP and 0.3 up to 0.5 μCi of γ-^{32}P-ATP. The tubes were incubated for 30 min at 34°C, and aliquots (100 μl) of reaction buffer were periodically withdrawn from each tube for hydrolysed ^{32}P assay. Hydrolysed ^{32}P was measured by scintillation counting of labeled ATP by addition of five volumes of 5% trichloroacetic acid containing 10% activated charcoal and 1-mM NaH_2PO_4. For determination of the ouabain-sensitive portion of total-ATPase activity, ^{32}P released in complete buffer for each group was compared to the activity of identically treated slices incubated in reaction buffer containing 3-mM ouabain. This latter, ouabain-insensitive ATPase (Mg^{2+}-ATPase) was subtracted from that found earlier (total ATPase) and the activity was corrected for protein content, determined in the homogenised tissue by colorimetric (Biorad, Melville, NY) assay. Under the incubation conditions used for the ATPase reaction, Na^+, K^+ and Mg^{2+} were saturating and basal Na^+,K^+-ATPase was optimised.

2.3. Cyclic GMP measurement

The content of cyclic GMP in the supernatants was determined by radioimmunoassay after acetylation of the samples (DuPont–New England Nuclear, Boston, MA). Preliminary experiments indicated that the amount of cyclic GMP released after the permeabilization procedure was equivalent to that released after homogenisation of slices.

2.4. Determination of nitrite / nitrate

Sodium nitroprusside (100 μM) was added (10 μl) to tubes containing 990-μl aliquots of the tissue preparation (five replicates), incubated for 15 min at 34°C, and then rapidly frozen on dry ice to stop the reaction. Samples were thawed, homogenised, centrifuged at $3700 \times g$ for 15 min and the supernatants were removed. NO production was determined indirectly by quantification of the nitrates/nitrites levels, as described by Green et al. (1982). In brief, samples (50 μl) were incubated with the same volume of Griess reagent at room temperature for 10 min. Absorbency was determined with an ELISA Multiskan

microplates reader at 550 nm. The results were plotted on a standard curve generated by analysing standards to which nitrate and nitrite had been added in known ratios.

2.5. Reagents

Routine reagents, sodium nitroprusside and ouabain were obtained from Sigma (St. Louis, MO). ATP tetra-(triethylamonium) salt (G-^{32}P) (6000 Ci/mmol) was purchased from DuPont–New England Nuclear. 8-bromo-cyclic GMP and okadaic acid were obtained from Research Biochemicals International (Natick, MA).

2.6. Statistics

All data are presented as means ± SEM of three independent experiments. In each experiment, five replicates were quantified. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test (Snedecor and Cochran, 1967). All $P < 0.05$ were considered to reflect a statistically significant difference.

3. Results

3.1. Age-related changes in Na^+,K^+-ATPase and Mg^{2+}-ATPase activity of proximal rat trachea

The Na^+,K^+-ATPase and Mg^{2+}-ATPase activities in control groups were similar in segments from 4- and 12-month-old animals but decreased in proximal trachea isolated from 24-month-old animals (Fig. 1a–c).

3.2. Effects of NO-donors on Na^+,K^+-ATPase and Mg^{2+}-ATPase activity, and cyclic GMP and nitrates/nitrites levels in proximal rat trachea

The NO-donor (sodium nitroprusside — 100 μM) significantly increased Na^+,K^+-ATPase activity in the proximal trachea of either 4- or 12-month-old rats, but did not change the activity of the enzyme of 24-month-old animals (Fig. 2a). On the other hand, Mg^{2+}-ATPase activity was not changed by sodium nitroprusside in tissue from any age studied (Fig. 2b).

Sodium nitroprusside was able to increase cyclic GMP and nitrites/nitrates level to a similar degree for all ages tested (Table 1). Therefore, although sodium nitroprusside did not activate Na^+,K^+-ATPase in the older group, this NO-donor was effective in increasing cyclic GMP and nitrites/nitrates levels. This finding suggested the hypothesis that some step downstream from cyclic GMP generation was impaired in the 24-month-old animals.

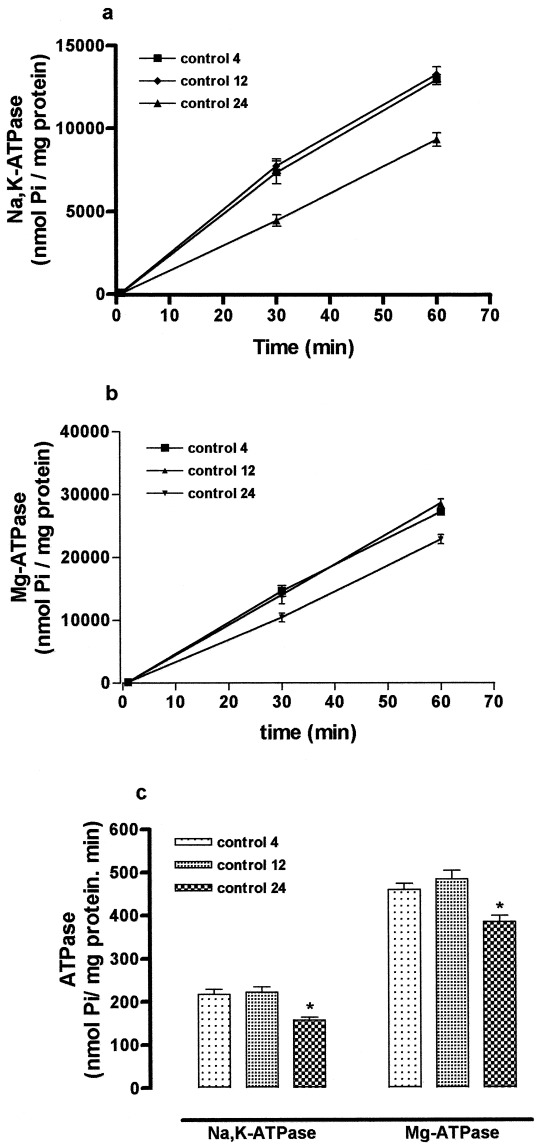

Fig. 1. Time course of the (a) Na^+,K^+-ATPase and (b) Mg^{2+}-ATPase in 4-, 12- and 24-month-old rat proximal trachea. The homogenised tissue from 4- and 12- month-old rats yielded (c) Na^+,K^+-ATPase activity of 197.8 ± 14.9 and 164.3 ± 11.8 nmol of Pi per mg of protein per minute, respectively, while in old (24-month-old) rats it was 105.1 ± 3.4 nmol of Pi per mg of protein per minute. (c) The Mg^{2+}-ATPase activity was 403.8 ± 34.2 nmol of Pi per mg of protein per minute in 4-month-old animals and of the 390.6 ± 28.0 nmol of Pi per mg of protein per minute in 12-month-old animals, while in old (24-month-old) rats it was 272.7 ± 24.3 nmol of Pi per mg of protein per minute. Both Na^+,K^+-ATPase and Mg^{2+}-ATPase activity from all ageing groups (24-month-old animals) were different from those in 4- and 12-month-old animals. Statistical analysis — Na^+,K^+-ATPase: *control-4 = **control-12 > control-24; Mg^{2+}-ATPase: **control-4 = **control-12 > control-24, $*P < 0.01$; $**P < 0.05$.

3.3. Effects of 8-bromo-cyclic GMP on the Na^+,K^+-ATPase and Mg^{2+}-ATPase activity of proximal rat trachea

Na^+,K^+-ATPase activity was measured in proximal segments of trachea from 4-, 12- and 24-month-old rat

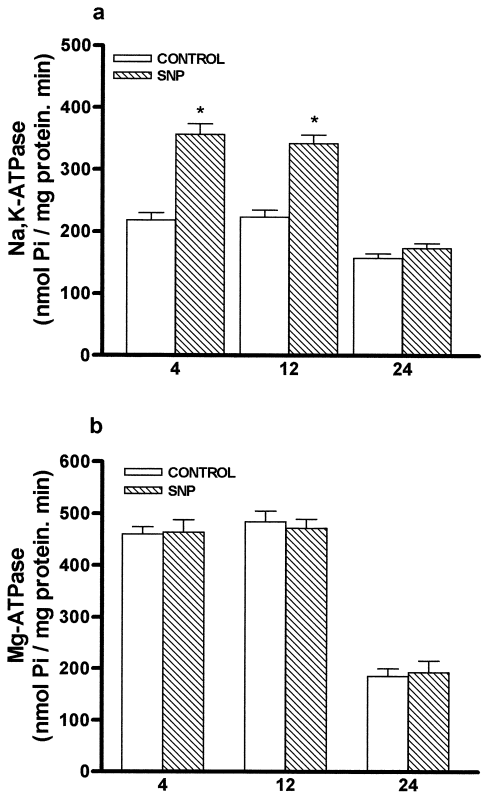

Fig. 2. Effects of sodium nitroprusside (SNP-100 μM) on Na^+,K^+-ATPase and Mg^{2+}-ATPase activity in proximal rat trachea from 4-, 12- and 24-month-old animals. Homogenates were incubated with each drug for 15 min at 34°C. Following incubation the drug was removed and ATPase was assayed. Mg^{2+}-ATPase activity was measured in the presence of 3 mM ouabain. Na^+,K^+-ATPase was determined from the difference between total ATPase and Mg^{2+}-ATPase activity. Statistical analysis — Na^+,K^+-ATPase: * SNP-4 = * SNP-12 > control-4 = control-12 > ** control-24 = ** SNP-24, * $P < 0.001$; ** $P < 0.05$.

trachea after incubation with 8-bromo-cyclic GMP (100 μM). Data showed that 8-bromo-cyclic GMP increased Na^+,K^+-ATPase activity in proximal segments of 4- and 12-month-old but not of 24-month-old rat trachea (Fig. 3a). On the other hand, 8-bromo-cylic GMP induced no change in Mg^{2+}-ATPase activity in proximal segments of rat trachea from the three ages studied (Fig. 3b).

Table 1
Effects of sodium nitroprusside (100 μM) on cyclic GMP and nitrites/nitrates levels in proximal rat trachea
Sodium nitroprusside was added to proximal segments and cyclic GMP and nitrites/nitrates levels determined. Values shown are mean ± SEM of triplicate samples, each assayed for cyclic GMP and nitrites/nitrates in duplicate.

Months	Control	Sodium nitroprusside
Cyclic GMP (fmol / mg)		
4	6.12 ± 0.42	9.42 ± 0.80[a]
12	5.86 ± 0.31	10.02 ± 0.56[a]
24	5.54 ± 0.23	9.54 ± 0.74[a]
Nitrites / nitrates (μM / mg)		
4	157.5 ± 11.5	221.2 ± 6.5[a]
12	160.1 ± 14.2	215.8 ± 6.5[a]
24	158.4 ± 9.3	206.7 ± 10.4[b]

[a] Statistical analysis compared to control: $P < 0.01$.
[b] Statistical analysis compared to control: $P < 0.05$.

3.4. Effect of inhibition of protein phosphatase on Na^+,K^+-ATPase and Mg^{2+}-ATPase activity of proximal rat trachea

To find if the aging process is linked to changes in phosphorylation mechanisms in the cyclic GMP modulation of Na^+,K^+-ATPase activity of proximal rat trachea we used okadaic acid, an inhibitor of protein phosphatase-1 and 2A activities (Cohen et al., 1990). Fig. 4a shows that incubation of the proximal rat trachea slices with okadaic acid (1 μM) increased proximal Na^+,K^+-ATPase activity in segments from 4-, 12- and 24-month-old animals. In addition, no change in Mg^{2+}-ATPase activity in proximal segments of rat trachea in the three different ages studied was obtained after incubation with okadaic acid (Fig. 4b). Therefore, no age-related changes were observed in a step

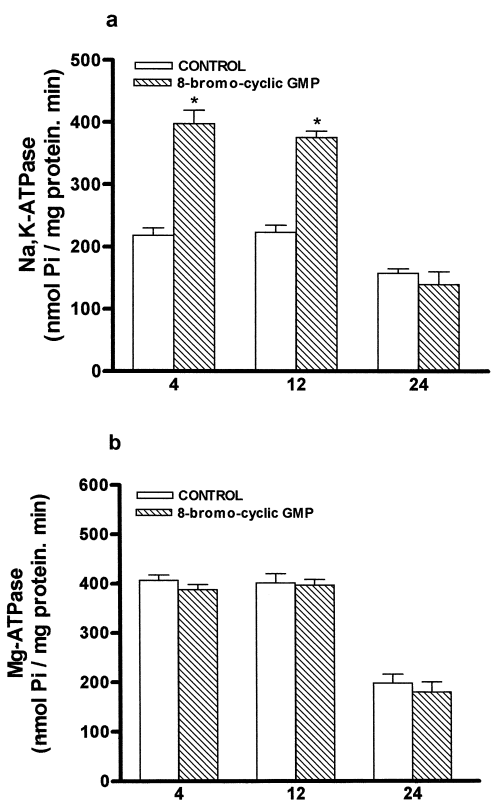

Fig. 3. Effects of 8-bromo-cyclic GMP (100 μM) on Na^+,K^+-ATPase and Mg^{2+}-ATPase activity in proximal rat trachea from 4-, 12- and 24-month-old animals. Homogenates were incubated with 8-bromo-cyclic GMP (100 μM) for 15 min at 34°C. Following incubation the drug was removed and ATPase was assayed. Mg^{2+}-ATPase activity was measured in the presence of 3 mM ouabain. Na^+,K^+-ATPase was determined from the difference between total ATPase and Mg^{2+}-ATPase activity. Statistical analysis — Na^+,K^+-ATPase: * 8Br-4 = * 8Br-12 > control-4 = control-12 > ** control-24 = ** 8Br-24, * $P < 0.001$; ** $P < 0.05$.

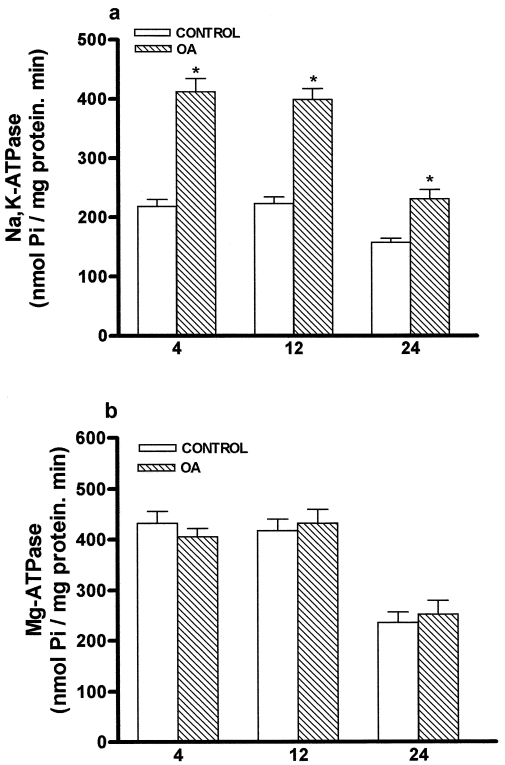

Fig. 4. Effects of OA (1 μM) on proximal Na^+,K^+-ATPase activity in rat trachea from 4-, 12- and 24-month-old animals. OA was dissolved in DMSO (0.1%) and samples were incubated (15 min at 34°C) with OA or vehicle (control). Mg^{2+}-ATPase activity was measured in presence of 3 mM ouabain. Statistical analysis — Na^+,K^+-ATPase: *OA-4 =*OA-12 > control-4 = control-12 = OA-24 > **control-24, $*P < 0.001$; $**P < 0.05$.

downstream from cyclic GMP-dependent protein kinase (i.e., phosphatase activity) acting through proximal rat trachea Na^+,K^+-ATPase.

4. Discussion

We have previously obtained evidence suggesting that Na^+,K^+-ATPase can be modulated by NO-cyclic GMP, cyclic GMP-dependent protein kinase and protein phosphatase in rat proximal trachea (De Oliveira Elias et al., 1999). We now studied whether proximal segments of rat trachea show a changing NO modulatory action on Na^+,K^+-ATPase activity through this intracellular pathway with aging.

The data from this work showed that trachea segments from 24-month-old animals exhibited a decrease in both Na^+,K^+-ATPase and Mg^{2+}-ATPase activities when compared to those from 4- and 12-month-old animals (Fig 1a–c). The reduction in Na pump activity could be due either to the age-related reduction in ATP levels (Hoyer, 1985) or to changes in the composition of the cell membrane due to lipoperoxidation (Chan et al., 1983; Viani et al., 1991; Tanaka and Ando, 1992). In addition, age-related changes in the expression of Na^+,K^+-ATPase α1- and α3-isoform mRNAs have been reported for rat hippocampus and cerebral cortex (Chauhan and Siegel, 1996, 1997). Therefore, we cannot rule out the possibility that changes in α- or β-Na^+,K^+-ATPase isoform expression might occur in proximal trachea and represent a candidate for an age-related factor predisposing to certain chronic inflammatory diseases.

It has been shown that the age-associated decrease in vasodilation induced by sodium nitroprusside, adenosine triphosphate, or adenosine is linked to a reduction of guanylate cyclase activity itself or at step(s) distal to this enzyme, such as cyclic GMP-phosphodiesterase in vascular smooth muscle (Ueda and Morikoti, 1991). The present study provides indirect evidence for age-related alterations in the Na^+,K^+-ATPase of proximal rat trachea by an action on cyclic GMP-dependent protein kinase. This effect is not restricted to trachea since decreased relaxation in response to sodium nitroprusside has been reported for longitudinal muscle strips of the gastric fundus from 24-month-old rats (Smits and Lefebvre, 1995).

Our data (Fig. 2a) showed that the stimulation of Na^+,K^+-ATPase in response to sodium nitroprusside was absent in trachea of aged (24 months) rats suggesting a loss of modulatory influence of this pathway in these animals. With regard to the activation of guanylate cyclase by NO, the increase in either cyclic GMP or nitrite/nitrate levels induced by sodium nitroprusside (Table 1) was similar in tissue from all three age groups studied (4-, 12- and 24-month-old animals). Therefore, the lack of a Na^+,K^+-ATPase modulatory response to sodium nitroprusside with aging is not linked to changes in guanylyl cyclase activity or to a differential NO release pattern from sodium nitroprusside in 24-month-old animals, but is probably related to a step downstream from cyclic GMP, such as cyclic GMP-dependent protein kinase. Furthermore, no differences in control cyclic GMP levels were observed in trachea segments from 24-month-old rats as compared with levels found in trachea isolated from either 4- or 12-month-old animals (Table 1). Therefore, there is no relationship between cyclic GMP levels and the reduction in Na^+,K^+-ATPase found in old animals. Further support for the notion that cyclic GMP-dependent protein kinase is a possible site modified by aging came from studies that showed an age-related decrease in relaxation response in the rat gastric fundus on treatment with zaprinast, an inhibitor of cyclic GMP-specific phosphodiesterase activity (Smits and Lefebvre, 1995). The results suggest an age-dependent change in enzyme activity that is compatible with an increase instead of a decrease in cyclic GMP levels in longitudinal muscle strips of the gastric fundus.

However, the most compelling evidence for a cyclic GMP-dependent protein kinase deficit in proximal trachea with aging is observations that 8-bromo-cyclic GMP stimulates rat proximal trachea Na^+,K^+-ATPase at 4- and 12-month-old animals, but not at 24 months. These results suggest that aging affects the cyclic GMP modulatory

pathway at the level of cyclic GMP-dependent protein kinase-dependent phosphorylation process. To further investigate this hypothesis, we used okadaic acid, a protein phosphatase inhibitor that enhances the net phosphorylated state of several intracellular proteins (Walaas and Greengard, 1991). The previously observed stimulation of Na^+,K^+-ATPase induced by okadaic acid in 4-month-old animals was also obtained with tissue from both 12- and 24-month-old animals when compared to the respective control groups. Therefore, these results suggest that the decreased response to nitroprusside and 8-bromo-cyclic GMP of proximal rat trachea with aging is not due to increased protein phosphatase activity, as the stimulatory effect of okadaic acid on Na pump activity should have decreased with age had this been the case (De Oliveira Elias et al., 1999).

Studies with cerebellum and hippocampus suggest that aging affects the NMDA receptor/NO synthase/cyclic GMP pathway (Vallebuona and Raiteri, 1995). These authors also reported no significant age-related changes in the basal extracellular levels of cyclic GMP in cerebellum, suggesting again that the cyclic GMP downstream step is probably more susceptible to aging. It is interesting to note that recent reports have suggested that NMDA receptors are present in the airways (Inagaki et al., 1995; Said et al., 1996). Glutamate production during a pathological process has been considered a defense mechanism and it might have a role in senescence. According to some authors, the stimulation of Na^+,K^+-ATPase activity by glutamate in the brain occurs through a carbon monoxide(CO)/NO-cyclic GMP and cyclic GMP-dependent protein phosphorylation cascade (Nathanson et al., 1995). Recent studies suggest that the cyclic AMP pathway is also modified in the aging process in both peripheral and central nervous systems (Smits and Lefebvre, 1995; Asanuma et al., 1996). Thus, it is conceivable that the convergence of several different pathways (such as: cyclic AMP/cyclic AMP-dependent protein kinase and cyclic GMP/cyclic GMP-dependent protein kinase) onto Na^+,K^+-ATPase activity would represent integrated effects of the different inputs, to allow a more sophisticated control of cellular function in the aging process.

The present study indicates with indirect evidence that the stimulation of cyclic GMP-dependent protein kinase by cyclic GMP is altered in aging animals, leading to a loss of stimulation of Na^+,K^+-ATPase by the NO-cyclic GMP pathway. Given that homogenised trachea tissue was used, it is impossible to know if age-related changes in cyclic GMP-dependent protein kinase occur in epithelial cells or in tracheobronchial smooth muscle. However, age-related changes in the NO-cyclic GMP pathway also occur for glutamate stimulation of rat cerebellar Purkinje $\alpha_3 Na^+,K^+$-ATPase (data not shown), suggesting that this effect is not restricted to proximal trachea and longitudinal muscle strips of the gastric fundus but appears to represent the failure of several important mechanisms that involve cyclic GMP-dependent protein kinase activity in both central and peripheral nervous systems of aging animals. This suggestion is also supported by the observation that several inhibitors of cyclic GMP-dependent protein kinase blocked senescence induced by inactivation of T antigen in SV-40-transformed immortal human fibroblasts (Fujii et al, 1995). Therefore, taken together, these results suggest that the change in the cyclic GMP pathway is involved in the induction of cellular senescence.

Therefore, it is reasonable to believe that the cyclic GMP-dependent protein kinase regulation of ciliary beat frequency (Wyatt et al 1998) and mucin secretion (Fischer et al 1999) in airway epithelial cells, as well as relaxation of the tracheobronchial smooth muscle (Zhou et al 1996) could be affected by the aging process. Thus, it would be interesting to measure cyclic GMP-dependent protein kinase activity in both tracheal epithelium and trachealis muscle separately for various age groups.

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References

Arystarkhova, E., Sweadner, K.J., 1997. Tissue-specific expression of the Na, K-ATPase β3 subunit. J. Biol. Chem. 272, 22405–22408.

Asanuma, M., Nishibayashi, S., Iwata, E., Kondo, Y., Nakanishi, T., Vargas, M.G., Ogawa, N., 1996. Alteration of cAMP response element-binding activity in the aged rat brain in response to administration of rolipram, a cAMP-specific phosphodiesterase inhibitor. Mol. Brain. Res. 41, 210–215.

Barnes, P.J., Belvisi, M.G., 1993. Nitric oxide and lung disease. Torax 48, 1034–1043.

Beal, M.F., Hyman, B.T., Koroshetz, W., 1993. Do effects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases?. Trends Neurosci. 16, 125–130.

Belvisi, M.G., Stretton, C.D., Yacoub, M.H., Barnes, P.J., 1992. Nitric oxide is the endogenous neurotransmitter of bronchodilator nerve in humans. Eur. J. Pharmacol. 210, 221–222.

Belvisi, M.G., Ward, J.A., Mitchell, J.A., Barnes, P.J., 1995. Nitric oxide as a neurotransmitter in human airways. Arch. Int. Pharmacodyn. Ther. 329, 97–110.

Besirli, C.G., Gong, T.W., Lomax, M.I., 1997. Novel β3 isoform of the Na,K-ATPase β subunit from mouse retina. Biochim. Biophys. Acta 1350, 21–26.

Buga, G.M., Gold, M.E., Wood, K.S., Chaudhuri, G., Ignarro, L.J., 1989. Endothelin-derived nitric oxide relaxes nonvascular smooth muscle. Eur. J. Pharmacol. 161, 61–72.

Chan, P.H., Kerlan, R., Fishman, R.A., 1983. Reduction of γ-amino-

butyric acid and glutamate uptake and Na^+,K^+-ATPase activity in brain slices and synaptossomes by arachidonic acid. J. Neurochem. 40, 309–316.

Chauhan, N., Siegel, G., 1997. Na,K-ATPase: increases in α1-messenger RNA and decrease in α3-messenger RNA levels in aging rat cerebral cortex. Neuroscience 78, 7–11.

Chauhan, N.B., Siegel, G.J., 1996. In situ analysis of Na,K-ATPase α1- and α3-isoform mRANs in aging rat hippocampus. J. Neurochem. 66, 1742–1751.

Cimino, M., Marini, P., Colombo, S., Cattabeni, F., Meldolesi, J., 1993. [^{3}H]-CGP 39653 mapping of glutamatergic *N*-methyl-D-aspartate receptors in the brain of aged rats. Neurosci. Res. Commun. 12, 31–39.

Cohen, P., Holmes, C.F.B., Tsukitani, Y., 1990. Okadaic acid: a new probe for the study of cellular regulation. Trends Biochem. Sci. 15, 98–102.

De Oliveira Elias, M., Tavares de Lima, W., Vannuchi, Y.B., Marcourakis, T., Da Silva, Z.L., Trezena, A.G., Scavone, C., 1999. Nitric oxide modulates Na^+,K^+-ATPase activity through cyclic GMP pathway in proximal rat trachea. Eur. J. Pharmacol. 367, 307–314.

Ellis, J.L., Undem, B.J., 1992. Inhibition by L-N^G-nitro-arginine of noradrenergic non-cholinergic relaxation of human isolated central and peripheral airways. Am. Rev. Respir. Dis. 146, 1543–1547.

Escolar, J.D., Gallego, B., Escolar, M.A., Minana, C., Roche, M., 1995. Age and experimental obstructive emphysema. A morphometrical study on the rat. Histol. Histopathol. 10, 875–887.

Fischer, B.M., Rochelle, L.G., Voynow, J.A., Akley, N.J., Adler, K.B., 1999. Tumor necrosis factor-alpha stimulates mucin secretion and cyclic GMP production by guinea pig tracheal epithelial cells in vitro. Am. J. Respir. Cell Mol. Biol. 20, 413–422.

Folkerts, G., Van Der Linde, H., Verheyen, A.K.C.P., Nijkamp, F.P., 1995. Endogenous nitric oxide modulation of potassium-induced changes in guinea-pig airway tone. Br. J. Pharmacol. 115, 1194–1198.

Fujii, M., Ide, T., Wadhwa, R., Tahara, H., Kaul, S.C., Mitsui, Y., Ogata, T., Oishi, M., Ayusawa, D., 1995. Inhibitors of cGMP-dependent protein kinase block senescence induced by inactivation of T antigen in SV40-transformed immortal human fibroblasts. Oncogene 11, 627–634.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of Nitrate, Nitrite, and [^{15}N] Nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Gruetter, C.A., Childers, C.E., Bosserman, M.K., Lemke, S.M., Ball, J.G., Valentovic, M.A., 1989. Comparison of relaxation induced by glyceryl trinitrate, isosorbide dinitrate, and sodium nitroprusside in bovine airways. Am. Rev. Respir. Dis. 139, 1192–1197.

Hoyer, S., 1985. The effect of age and glucose and energetic metabolism in brain cortex of rats. Arch. Gerontol. Geriatr. 4, 193–203.

Inagaki, N., Kuromi, H., Gonoi, T., Okamoto, Y., Ishida, H., Seino, Y., Kaneko, T., Iwanaga, T., Seino, S., 1995. Expression and role of ionotropic glutamate receptors in pancreatic islet cells. FASEB J. 9, 686–691.

Katsuki, S., Murad, F., 1977. Regulation of adenosine cyclic 3′,5′-monophosphate and guanosine cyclic 3′,5′-monophosphate levels and contractility in bovine tracheal smooth muscle. Mol. Pharmacol. 13, 330–341.

Li, C.G., Rand, M.J., 1991. Evidence that part of the NANC relaxant response of guinea-pig trachea to electrical field stimulation is mediated by nitric oxide. Br. J. Pharmacol. 102, 91–94.

Lipton, S.A., 1994. AIDS-related dementia and calcium homeostasis. Ann. N.Y. Acad. Sci. 747, 205–224.

McCann, S.M., 1997. The nitric oxide hypothesis of brain aging. Exp. Gerontol. 32, 431–440.

McCann, S.M., Licinio, J., Wong, M.L., Yu, W.H., Karanth, S., Rettorri, V., 1998. The nitric oxide hypothesis of aging. Exp. Gerentol. 33, 813–826.

Murad, F., Mittal, C.K., Arnold, W.P., Katsuki, S., Kimura, H., 1978. Guanylate cyclase: activation by azide, nitrocompounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. Adv. Cyclic Nucleotide Res. 9, 145–158.

Nathanson, J.A., Scavone, C., Scanlon, C., Mckee, M., 1995. The cellular Na^+ pump as a site of action for carbon monoxide and glutamate: a mechanism for long-term modulation of cellular activity. Neuron 14, 781–794.

Nijkamp, F.P., Folkerts, G., 1994. Nitric oxide and bronchial reactivity. Clin. Exp. Allergy 24, 905–914.

Nijkamp, F.P., Folkerts, G., 1995. Nitric oxide and bronchial hyperresponsiveness. Arch. Int. Pharmacodyn. Ther. 329, 81–96.

Peterson, C., Cotman, C.W., 1989. Strain-dependent decrease in glutamate binding to the *N*-methyl-D-aspartic acid receptor during aging. Neurosci. Lett. 9, 309–313.

Renwick, D.S., Connolly, M.J., 1999. Improving outcomes in elderly patients with asthma. Drugs Aging 14, 1–9.

Rossi, A., Ganassini, A., Tantucci, C., Grassi, V., 1996. Aging and the respiratory system. Aging (Milano) 8, 143–161.

Said, S.I., Berisha, H.I., Pakbaz, H., 1996. Excitotoxicity in the lung: *N*-methyl-D-aspartate-induced, nitric oxide-dependent, pulmonary edema is attenuated by vasoactive intestinal peptide and by inhibitors of poly(ADP-ribose)-polymerase. Proc. Natl. Acad. Sci. U.S.A. 93, 4688–4692.

Smits, G.J.M., Lefebvre, R.A., 1995. Influence of age on the signal transduction pathway of non-adrenergic non-cholinergic neurotransmitters in the rat gastric fundus. Br. J. Pharmacol. 114, 640–647.

Snedecor, G., Cochran, W., 1967. One way classifications-analysis of variance. In: Snedecor, G.W., Cochran, W.G. (Eds.), Statistical Methods. 6th edn. Iowa State University Press, Ames, pp. 258–298.

Sweadner, K.J., 1989. Isozymes of the Na^+/K^+-ATPase. Biochim. Biophys. Acta 988, 185–220.

Tamaru, M., Yoneda, Y., Ogita, K., Shimizu, J., Nagata, Y., 1991. Age-related decreases of the *N*-methyl-D-aspartate receptor complex in the rat cerebral cortex and hippocampus. Brain Res. 542, 83–90.

Tanaka, Y., Ando, S., 1992. Age-related changes in [^{3}H] ouabain binding to synaptic plasma membrane isolated from mouse brains. J. Biochem. (Tokyo) 112, 117–121.

Torphy, T.J., 1994. β-Adrenoceptors, cAMP and airway smooth muscle relaxation: challenges to the dogma. Trends Pharmacol. Sci. 15, 370–374.

Ueda, H., Morikoti, H., 1991. Possible association of decrease of ATP-induced vascular relaxation with reduction of cyclic GMP during aging. Arch. Int. Pharmacodyn. Ther. 310, 35–45.

Vallebuona, F., Raiteri, M., 1995. Age-related changes in the NMDA receptor/nitric oxide/cGMP pathway in the hippocampus and cerebellum of freely moving rats subjected to transcerebral microdialysis. Eur. J. Neurosci. 7, 694–701.

Viani, P., Cervato, G., Fiorilli, A., Cestaro, B., 1991. Age-related differences in synaptosomal peroxidative damage and membrane properties. J. Neurochem. 56, 253–258.

Walaas, S.I., Greengard, P., 1991. Protein phosphorylation and neuronal function. Pharmacol. Rev. 43, 299–349.

Wenk, G.L., Walker, L.C., Price, D.L., Cork, L.C., 1991. Loss of NMDA, but not GABA-A, binding in the brains of aged rats and monkeys. Neurobiol. Aging 12, 93–98.

Wyatt, T.A., Spurzem, J.R., May, K., Sisson, J.H., 1998. Regulation of ciliary beat frequency by both PKA and PKG in bovine apithelial cells. Am. J. Physiol. 275, L827–L835.

Zhou, X.B., Ruth, P., Schlossmann, J., Hofmann, F., Korth, M., 1996. Protein phosphatase 2A is essential for the activation of Ca^{2+}-activated K^+ currents by cGMP-dependent protein kinase in tracheal smooth muscle and Chinese hamster ovary cells. J. Biol. Chem. 271, 19760–19767.